THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 268, No. 13, Issue of May 5, pp. 9753-9761, 1993
Printed in U.S.A.

Molecular Analysis of Human β-Arrestin-1: Cloning, Tissue Distribution, and Regulation of Expression

IDENTIFICATION OF TWO ISOFORMS GENERATED BY ALTERNATIVE SPLICING*

(Received for publication, October 20, 1992)

Giustino Parruti‡, Francesca Peracchia, Michele Sallese, Grazia Ambrosini, Mariateresa Masini, Domenico Rotilio, and Antonio De Blasi§

From the Consorzio Mario Negri Sud, Istituto di Ricerche Farmacologiche Mario Negri, 66030 Santa Maria Imbaro, Italy

The cDNA for human β-arrestin-1 was cloned by polymerase chain reaction (PCR) and identified based on its remarkably high amino acid identity (98.6%) with the bovine sequence. Two alternatively spliced isoforms of human β-arrestin-1, differing only in the presence or absence of 24 base pairs/8 amino acids within the sequence, were identified and called β-arrestin-1A and β-arrestin-1B, respectively. Both isoforms were found in all tissues tested. Southern blot analysis revealed the existence of a single gene for β-arrestin-1, suggesting that the two isoforms are generated by alternative mRNA splicing. The possible presence of similar isoforms was investigated for the other members of the arrestin/β-arrestin gene family by PCR. Two isoforms of arrestin were revealed in bovine peripheral blood leukocytes. The expression of β-arrestin-1 was studied in several human tissues and cell types. High levels of β-arrestin-1 mRNA and immunoreactivity were found in peripheral blood leukocytes. The possible regulation of the expression of β-arrestin-1 was also investigated. Our work documents for the first time that the expression of β-arrestin-1 is modulated by intracellular cAMP. Using two cell types, human endothelial cells and smooth muscle cells, we found that 6–8-h treatments with the cAMP-inducing agents cholera toxin, forskolin, iloprost, and isoproterenol raised β-arrestin-1 mRNA by 2–4-fold. Forskolin preferentially increased β-arrestin-1A expression in smooth muscle cells, as assessed by PCR. β-Arrestin-1 immunoreactivity was 2–3-fold higher in smooth muscle cells exposed to forskolin for 8 h, compared with untreated controls.

We conclude that (i) the finding of alternatively spliced isoforms of β-arrestin-1 and arrestin documents a novel mechanism to generate diversity within the arrestin/β-arrestin gene family; (ii) the abundant expression of β-arrestin-1 in peripheral blood leukocytes further supports our previous suggestion of a major role for the βARK/β-arrestin system in regulating receptor-mediated immune functions; (iii) the increased expression of β-arrestin-1 by cAMP suggests a new mechanism for the regulation of receptor-mediated responses.

The responsiveness of many receptors to their own stimuli can be acutely regulated. In particular, for a number of receptors a rapid, drastic, and reversible loss of responsiveness has been shown to occur upon exposure to agonists (1–3). This phenomenon is known as homologous desensitization and has been best characterized on the model of the β_2-adrenergic receptor (βAR)[1] (1–3). Two types of proteins have been shown to play a major role in determining homologous desensitization: β-adrenergic receptor kinase (βARK) (4, 5) and its functional cofactor, β-arrestin (6–9). βARK has been shown to phosphorylate βAR and other G-coupled receptors rapidly when they are occupied by their agonists (1–5). The family of such receptor kinases, as identified by molecular cloning, includes rhodopsin kinase (10), βARK-1 (4, 5), and βARK-2 (11).

β-Arrestin is a cytosolic protein essential for complete desensitization of phosphorylated receptors *in vitro* (6–8). So far three members of the arrestin/β-arrestin gene family have been identified. Arrestin is the protein that regulates phototransduction in the retina. Its primary structure is now known in several species, including human (12) and bovine (13). More recently two subtypes of β-arrestin were identified by molecular cloning. The cDNA of β-arrestin-1 was cloned from bovine (6) and rat (8), whereas β-arrestin-2 was cloned from human (9) and rat (8). β-Arrestin-1 and β-arrestin-2 were equipotent in inhibiting βAR function (8). Conversely, arrestin inhibited rhodopsin coupling to trasducin, whereas β-arrestin-1 and β-arrestin-2 were at least 20-fold less potent in this system (8).

βARK-1, βARK-2, β-arrestin-1, and β-arrestin-2 were all reported as preferentially expressed in the central nervous system, and a specific role in the regulation of synaptic receptors, exposed to high concentrations of agonist, was suggested (1–4, 6, 8, 11). We have shown recently that peripheral blood leukocytes (PBL) represent a major site of βARK

* This work was supported in part by a grant from P. F. Ingegneria Genetica, Consiglio Nazionale delle Ricerche, and by the Agenzia per la Promozione dello Sviluppo del Mezzogiorno (del. N.6166, PR 1). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L04685.

‡ Recipient of a fellowship from the Centro di Formazione e Studi per il Mezzogiorno (FORMEZ), progetto speciale Ricerca Scientifica e Applicata nel Mezzogiorno.

§ To whom correspondence should be addressed: Consorzio Mario Negri Sud, via Nazionale, 66030 S. Maria Imbaro, Italy. Tel.: 39-872-570-351; Fax: 39-872-578-240.

[1] The abbreviations used are: βAR, β-adrenergic receptors; βARK, β-adrenergic receptor kinase; PBL, peripheral blood leukocytes (granulocytes + lymphocytes + monocytes); PCR, polymerase chain reaction; bp, base pair(s); MNL, mononuclear leukocytes (lymphocytes + monocytes); F, forward primer; R, reverse primer; G_s, stimulatory guanine nucleotide-binding regulatory protein; SMC, smooth muscle cells; HEC, human umbilical vein endothelial cells; kb, kilobase(s).

expression and function (5, 11). In these cells, we found that mRNA expression and kinase activity of βARK-1 and βARK-2 were as high as in the brain. Furthermore, the β-adrenergic agonist isoproterenol and platelet-activating factor, which in these cells play a significant role in regulating immune functions, were able to induce βARK activation (5). These data suggest that βARK may be a potent modulator of receptor-mediated immune functions (5, 11) and raise the possibility of a relevant role for β-arrestin as well.

In the present study, the cDNA for human β-arrestin-1 was cloned, and two molecular forms generated by alternative mRNA splicing were identified. β-Arrestin-1 expression was found to be high in immune cells and regulated by cAMP in two cellular models.

EXPERIMENTAL PROCEDURES

Human and Bovine Tissue and Cell Sources—Macroscopically normal tissues, obtained from surgically excised samples, were rapidly frozen in liquid nitrogen. Heart samples were myocardial specimens from hearts explanted for idiopathic congestive cardiomyopathy; lung, liver, and breast samples were taken 5–8 cm away from the periphery of tumor tissue. Skeletal muscle and adipose tissue specimens were from rectum abdominis and subcutaneous soft tissues of laparotomized patients. Bovine tissues were collected in the local slaughterhouse. To prevent RNA degradation, tissue samples were thawed and minced directly in guanidine isothiocyanate. Cultured cells (American Tissue Culture Collection) were grown under standard conditions (5) and harvested directly in guanidine isothiocyanate. PBL were fractionated as described previously (5, 14). RNA samples were checked for degradation and discarded if partially degraded.

Polymerase Chain Reaction (PCR) Cloning of Human β-Arrestin-1—The cloning strategy was as reported previously (5). When this study was planned, the cloning of bovine β-arrestin-1 had not been yet reported. Based on the sequence of bovine arrestin (13) we then designed oligonucleotides F2 (bp 682–701 of the coding region as in Ref. 13) and R4 (bp 894–874, Ref.13) and obtained a PCR product that turned out to correspond to bp 684–855 of human β-arrestin-1 (numbered starting at the beginning of the coding region, *i.e.* base 1 is the A of ATG). To clone the entire coding region, we used primers F1 and R1 (bp 1–23 and bp 1254–1231, respectively, of the coding sequence of bovine β-arrestin-1, Ref. 6) and primers F3 (bp 734–755), F4 (bp 958–980), R2 (bp 1144–1121), R3 (bp 1111–1092), and R5 (bp 853–832) designed on the human sequence obtained from previously sequenced PCR products. The cDNA fragments cloned are: F2-R4, F1-R5, F3-R1, F4-R2, and F4-R3. PCR reactions were carried out as described before (5) with minor modifications. To obtain the first cDNA strand, 1 μg of total RNA (in one case poly(A)$^+$ RNA) from mononuclear leukocytes (MNL) and adipose tissue was reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase. PCR was performed for 36 cycles, with 1-min denaturation at 94 °C, 1-min annealing at various temperatures (Ref. 5), and 4-min extension at 72 °C. PCR products were subcloned blunt end in PTZ18R or Bluescript and sequenced in both directions.

Analysis of mRNA Isoforms by PCR—Total RNA was reverse transcribed as described above. For β-arrestin-1, primers F4, R2, and R3 were designed to amplify cDNA fragments F4-R2 (bp 958–1144) and F4-R3 (bp 958–1111), both encompassing the site of alternative mRNA splicing. F4 and R2, which were designed on regions identical in human and bovine cDNAs, were used also for amplification of bovine RNA. The amplification conditions were: 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min for 30 cycles. PCR products were separated and visualized by gel electrophoresis using 3:1 NuSieve agarose at 4%. The nature of the amplification products was confirmed by restriction analysis and/or sequencing. When appropriate, Southern blot analysis of a 1-μl PCR reaction was performed using the labeled F4-R3 fragment as a probe. As the two isoforms of β-arrestin-1 differ only for the presence or absence of a 24-bp sequence, when amplified with a pair of oligonucleotides flanking that region (such as F4-R3), both forms represent indifferent templates for the PCR reaction (competitive PCR, Ref. 15). As a consequence, the relative abundance of these PCR products directly measures the ratio of the two transcripts at any cycle of amplification (15). The relative density of bands imprinted on the autoradiographic films was measured by laser densitometry (Pharmacia LKB Biotechnology Inc.).

For β-arrestin-2, a 270-bp portion of the human β-arrestin-2 cDNA was amplified. Primers F(t)1 and R(t)1 (bp 824–844 and bp 1093–1074, respectively) were designed on the sequence reported in Ref. 9. The amplification conditions used were: 94 °C for 1 min, 63 °C for 1 min, 72 °C for 1 min for 30 cycles. PCR products were visualized as described above.

For arrestin, a 169-bp portion of the bovine arrestin cDNA was amplified. Primers F(r)1 and R(r)1 (bp 940–959 and bp 1108–1088 of the coding sequence, respectively) were based on the sequence reported in Ref. 13. The amplification conditions used were: 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min for 30 cycles. PCR products were visualized as described above.

The components of PCR reactions were tested for contaminants by 40-cycle reactions without added DNA template.

Southern Blot Analysis of Genomic DNA—High molecular weight genomic DNA was prepared from human PBL. Cells were treated with 100 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% sodium dodecyl sulfate (SDS) and proteinase K (100 μg/ml) for 16 h at 37 °C, followed by phenol extraction (16). RNA was removed from the preparation by treatment with DNase-free RNase A (50 μg/ml) for 2 h at 37 °C, followed by a second phenol extraction. High molecular weight DNA (10 μg) was digested with each restriction endonuclease under conditions indicated by the manufacturer, fractionated by 0.8% agarose gel, and transferred to a Nylon membrane (GeneScreen Plus) (17). Filters were hybridized using as a probe the F2-R4 cDNA fragment of β-arrestin-1 labeled as described below, washed to a final stringency of 0.1 × SSC, 0.1% SDS at 65 °C, and subjected to autoradiography at −80 °C for 1–4 days. The hybridization signal was evident in all cases after a 1-day exposure, but a long-term exposure (4 days) was performed to reveal possible additional hybridization bands.

Northern Blot Analysis—To examine mRNA expression of human β-arrestin-1, the cDNA fragment bp 684–855 was used as a probe for Northern blot analysis. When this was labeled by random priming, the hybridization pattern revealed by autoradiography was barely detectable even after long term exposures (≥15 days) (18). Similar results were obtained using longer cDNA fragments as probes; also the use of poly(A)$^+$ RNA did not significantly improve the quality of results (data not shown). A substantial improvement was obtained when using as a probe antisense single-stranded DNA generated by Taq polymerase. Two to 5 ng of the described cDNA fragment was used as a template after gel purification. The reaction mixture (10 μl final volume) contained 15 pmol of dATP, dGTP and dTTP; 7.5 pmol of dCTP; 25 μCi (8.3 pmol) of [^{32}P]dCTP (3,000 Ci/mmol); 200 ng of the antisense primer R4, 1 μl of 10 × PCR buffer; and 2 units of Taq polymerase. The mixture was cycled for 35 cycles. Northern blot analysis of βARK-1 was performed as described previously (5), using the random primed cDNA fragment bp 1055–1946 as a probe. RNA blots were hybridized and washed as described (5) and subjected to autoradiography at −80 °C for 1–4 days. All results were confirmed at least in two separate experiments, using material from either different individuals or different cell treatments.

Western Blot Analysis—The monoclonal antibody F4C1 (19), raised against the highly conserved epitope DGVVLVD, identical in human β-arrestin-1 (amino acids 38–44 as in Fig. 1), β-arrestin-2, and arrestin, was kindly provided by Drs. K. Palczewski and L. A. Donoso. Cytosolic preparations from human tissues and cells were obtained as follows. Cells or tissue fragments were pelleted by centrifugation (800 × *g* for 5 min), lysed in cell lysis buffer (10 mM Tris, 5 mM EDTA, 7.5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μg/ml benzamidine, at pH 7.4) using a Polytron tissue disruptor (Janke and Kundel) at low speed for 40 s on ice. Unbroken cells and cell nuclei were pelleted by centrifugation (800 × *g* for 5 min) and discarded. The supernatant was then centrifuged at 300,000 × *g* for 30 min at 4 °C. The protein content of the resultant supernatant (cytosolic fraction) was measured using Bio-Rad protein assay reagent. Samples containing 100 μg of protein were suspended in Laemmli buffer (17) by gentle shaking for 5–10 min and electrophoresed on 12% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes with a tank transfer system. Efficiency of transfer was verified by Ponceau red staining of the blots and Coomassie Blue staining of gels after transfer. The blots were blocked overnight with 4% bovine serum albumin in Tris-buffered saline. β-Arrestin-1 was detected with the specific antibody followed by plus peroxidase-coupled second antibody using 5-chloro-1-naphthol as revealing substrate. The resulting blots were scanned four times with an LKB Ultroscan XL laser densitometer. All results were confirmed in at least two separate experiments, using material from either different individuals or different cell treatments.

Cell Treatments—Human umbilical vein endothelial cells (HEC) were isolated from the umbilical cord vein as described (20) and used within six *in vitro* passages (splitting ratio 1:3). Cells were grown on 1.5% gelatin in medium 199 with 20% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml endothelial cell growth supplement, and 50 μg/ml heparin in 5% CO_2 at 37 °C. For treatments, cells were grown to confluence in 100-mm Petri dishes, washed twice with serum-free medium 199, then incubated with medium 199 supplemented with 10% fetal calf serum without heparin and endothelial cell growth supplement, with or without stimuli. At the end of treatment, cells were directly harvested in guanidine isothiocyanate (21). Parallel control cells were similarly washed and harvested at all experimental time points. In time course experiments with HEC, untreated control cells for each time point were considered, as the culture conditions used during treatments affected the mRNA levels of the genes of interest.

Human smooth muscle cells (SMC) (5) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin in 5% CO_2 at 37 °C. Cells ~70% confluent were supplemented either with fresh culture medium (control untreated cells) or with medium containing the different drugs. Control cells were similarly washed and harvested at all experimental time points. Different times of incubation did not affect the mRNA levels of the genes of interest in untreated SMC.

Materials—Deoxynucleotides used for PCR and Amplitaq DNA polymerase were from Perkin-Elmer Cetus Instruments; Moloney murine leukemia virus reverse transcriptase, T7 DNA polymerase, other modifying enzymes and restriction endonucleases were from Bethesda Research Laboratories, Pharmacia, or Boehringer Mannheim. NuSieve 3:1 agarose was from FMC. GeneScreen Plus membranes were from Du Pont-New England Nuclear. ^{35}S- and ^{32}P-labeled dNTPs and the random priming kit were purchased from Amersham Corp. Gelatin was from Difco. Culture media, fetal calf serum, okadaic acid, guanidine isothiocyanate were from GIBCO BRL. Heparin (from porcine intestinal mucosa), proteinase K, phorbol 12-myristate 13-acetate, cycloheximide, and isoproterenol were from Sigma. Forskolin, ionomycin, and cholera toxin were from Calbiochem. Iloprost was kindly provided by Schering, Germany.

RESULTS

Cloning of Human β-Arrestin-1 cDNA and Identification of Two Isoforms—The human β-arrestin-1 cDNA was cloned and sequenced. The sequence displayed a very high similarity to the bovine β-arrestin-1 cDNA (6) (93% identity). Only 6 out of 418 deduced amino acids were different (amino acids 35, 146, 165, 229, 368, and 400 as in Fig. 1) resulting in 98.6% identity. Our sequence was identified as the human form of β-arrestin-1, based on this extremely high similarity. The human amino acid sequences of the three known members of the arrestin/β-arrestin gene family are aligned in Fig. 1.

During the analysis of PCR products F3-R1 (bp 734–1254), F4-R2 (bp 958–1144), and F4-R3 (bp 958–1111) we observed the existence of two molecular isoforms of human β-arrestin-1. Using F4-R2 and F4-R3, two amplification products were generated in each case, of 187 and 163 bp and of 154 and 130 bp, respectively. Two products were also generated with F3-R1, although they were poorly resolved by agarose gel electrophoresis (not shown). To clarify the nature of these doublets, we subcloned and sequenced both bands of the F4-R3 amplification. Sequence analysis revealed that the two fragments differed only by the presence or absence of 24 bp (bp 999–1022), whereas the flanking sequences were found to be identical in both bands. This portion of the human cDNA is presented in Fig. 1*B*, aligned with the corresponding regions of β-arrestin-2 and arrestin. The 24-bp sequence present or absent in the two isoforms encodes for an in-frame sequence of 8 amino acids (*boxed* in Fig. 1). The two isoforms were called β-arrestin-1A and β-arrestin-1B in the presence or absence of the 24-bp fragment, respectively. We also identified these two alternatively spliced forms in bovine RNA. PCR performed with F4 and R2 on RNA from bovine PBL generated two amplification products of 187 and 163 bp (not shown). The doublet was subcloned and sequenced. Sequence analysis revealed that the two fragments differed only by the presence or absence of 24 bp corresponding exactly to the site of splicing as in human β-arrestin-1 (Fig. 1*B*). This sequence was identical to that reported in Ref. 6, except for the absence of the 24 bp in the shorter form.

Genomic Southern Blot Analysis of Human β-Arrestin-1—We investigated whether β-arrestin-1 isoforms were generated by alternative mRNA splicing of a single gene or represented transcripts of different genes. The F2-R4 cDNA fragment of β-arrestin-1, which is identical in the two isoforms, was used to probe blots of human genomic DNA digested with several restriction endonucleases in single restriction experiments. This probe always revealed a single restriction fragment, even after long term (4 days) exposures of filters (Fig. 2). These results are compatible with the existence of a single gene for β-arrestin-1 in the human genome and suggest that the two isoforms are generated by alternative mRNA splicing.

Alternatively Spliced Isoforms within the Arrestin/β-Arrestin Gene Family—β-Arrestin-1 is one of the three known members of the arrestin/β-arrestin gene family. This prompted us to investigate whether the presence of the two alternatively spliced isoforms observed for human and bovine β-arrestin-1 could also be demonstrated for the other members of this gene family. For this purpose we used a pair of oligonucletides to amplify the regions corresponding to the site of the alternative mRNA splicing of β-arrestin-1 in each gene of interest. Primers were designed on regions of maximal divergence among these three cDNAs. The specificity of the PCR reactions for each gene was tested (not shown).

Such an analysis was performed for β-arrestin-2 using F(t)1 and R(t)1 as primers. A single PCR product of 270 bp was obtained in all amplifications (Fig. 3), corresponding to bp 834–1093 of the sequence reported in Ref. 9. The nature of this product was verified by both restriction analysis and sequencing. Southern blot analysis of 10 μl of the PCRs confirmed the presence of a single PCR product (not shown) revealing that, at least in the tissues considered, only a single molecular form of β-arrestin-2 is present.

To analyze arrestin transcripts we performed PCR on bovine RNA using F(r)1 and R(r)1 as primers. Bovine material was used, as human retinal specimens were not available. From bovine retinal RNA a single product of 169 bp was generated, corresponding to bp 940–1088 of the sequence reported in Ref. 13 (Fig. 3). The presence of a single product in this amplification was confirmed by Southern blot analysis of 15 μl of PCR (not shown). We then analyzed RNA from several non-retinal tissues (Fig. 3). PCR on RNA from lung and heart did not reveal any product, suggesting that arrestin is not expressed in these tissues. By contrast, one product of 169 bp was generated from cerebellum and brain, whereas two amplification products of 169 and 145 bp, respectively, were obtained from PBL RNA (Fig. 3). The doublet from PBL was subcloned and sequenced. Sequence analysis revealed that the two fragments differed only by the presence or absence of 24 bp, corresponding exactly to the site of splicing of human and bovine β-arrestin-1 (Fig. 1). By analogy with β-arrestin-1 we named these two isoforms arrestin A and arrestin B, in the presence or absence of the 24 bp, respectively.

Tissue Distribution of β-Arrestin-1 mRNA and Immunoreactivity—β-Arrestin-1 mRNA expression was studied using two complementary approaches: (i) the level of β-arrestin-1 mRNA was determined by Northern blot analysis; (ii) the relative amount of β-arrestin-1A and β-arrestin-1B was analyzed by PCR (competitive PCR, Ref. 15).

The β-arrestin-1 cDNA fragment of bp 684–855 was used

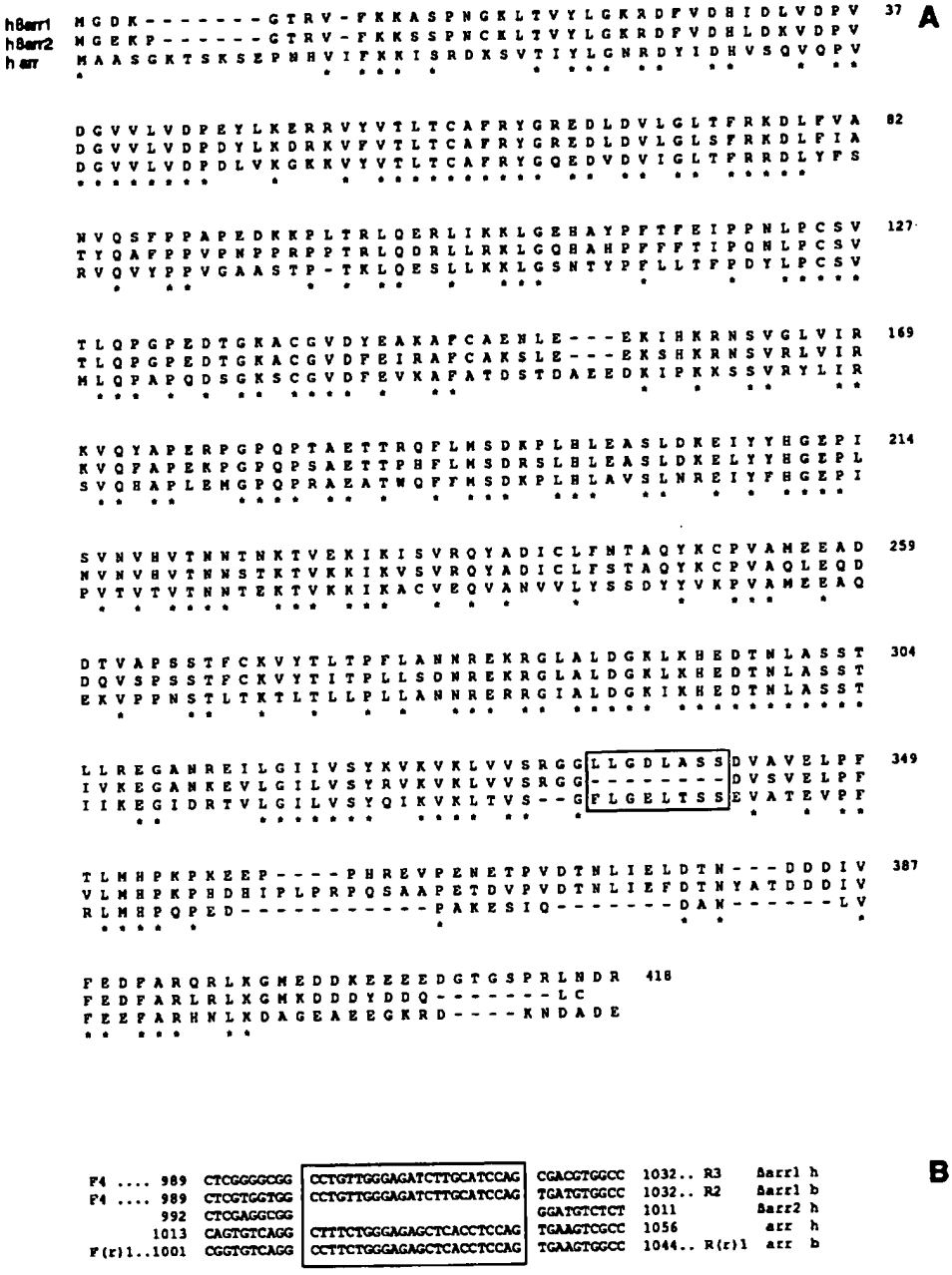

FIG. 1. **Deduced amino acid sequence and alternative splicing of human β-arrestin-1.** *Panel A*, alignment of amino acid sequences of human β-arrestin-1 (*h βarr1*), human β-arrestin-2 (*h βarr2*) (9), and human arrestin (*h arr*) (12). *Gaps* were introduced to obtain maximal homology using the sequence analysis software package from Genetics Computer Group, Inc. *Asterisks* indicate amino acids conserved among the three proteins. The alternatively spliced 8 amino acids are *boxed*. *Panel B*, alignment of the alternatively spliced cDNA sequence within the arrestin/β-arrestin gene family. The region encompassing the site of alternative splicing in human β-arrestin-1 (*βarr1 h*) was aligned with the sequences of human β-arrestin-2 (*βarr2 h*) and human arrestin (*arr h*). The sequence of bovine β-arrestin-1 (*βarr1 b*) was obtained from the PCR products F4-R2, whereas that of bovine arrestin (*arr b*) was obtained from the PCR products F(r)1-R(r)1. The *boxed sequences* are: the site of alternative mRNA splicing for human and bovine β-arrestin-1 and bovine arrestin; exon 13 for human arrestin gene; a gap for human β-arrestin-2.

as a probe for Northern blot analysis. The use of the labeling procedure modified by us as described under "Experimental Procedures" proved to be critical for these experiments. Three major mRNA species of 7.5, 3.0, and 1.7 kb in size were detected (Fig. 4). Such bands are presently interpreted as different products of mRNA processing (6, 18). The 7.5-kb species was the most abundant; in some cell types such as SMC (see below) and SW626 (18), it appeared as the only detectable message. We considered the abundance of the 7.5-kb band when comparing mRNA expression levels either in different tissues or after cell treatments.

Detectable amounts of β-arrestin-1 mRNA were found in all tested tissues. The highest levels of expression were observed in MNL and lung, whereas moderate to low mRNA levels were found in heart, skeletal muscle, and liver (Fig. 4). An abundant expression of β-arrestin-1 was also found in subcutaneous adipose tissue (not shown). One goal of our investigation was to compare β-arrestin-1 mRNA in PBL and

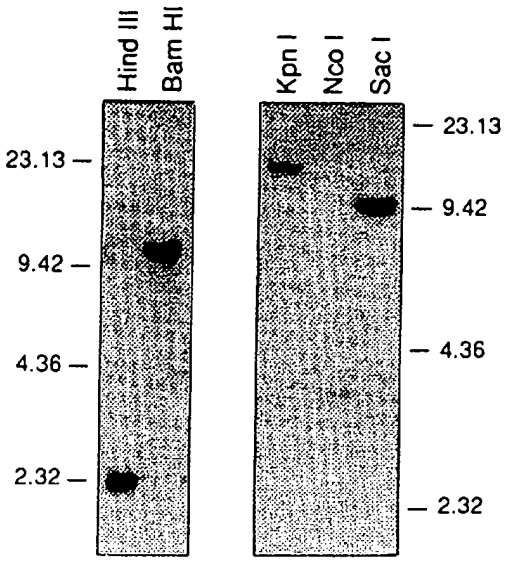

FIG. 2. **Southern blot analysis of human genomic DNA.** Large molecular size DNA (10 μg) was treated with the indicated restriction endonucleases, fractionated by a 0.8% agarose gel, transferred to a nylon membrane, and probed as described under "Experimental Procedures." Washed filters were exposed at −80 °C for 4 days. The molecular size (in kilobases) of standards is shown.

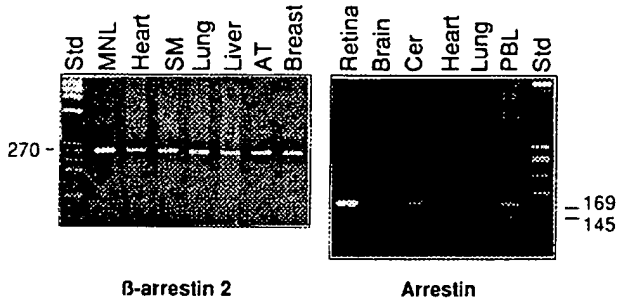

FIG. 3. **PCR analysis of β-arrestin-2 and arrestin mRNA.** *Left panel*, analysis of β-arrestin-2 mRNA from human MNL, heart, skeletal muscle (*SM*), lung, liver, adipose tissue (*AT*), and breast. PCR reactions (18 μl) were analyzed by electrophoresis in 4% NuSieve 3:1 agarose gel. The amplification product of these reactions (270 bp) is indicated. *Right panel*, analysis of arrestin mRNA from bovine retina, brain, cerebellum (*Cer*), heart, lung, and PBL. PCR (18 μl) were analyzed by electrophoresis in 4% NuSieve 3:1 agarose gel. The two amplification products of these reactions (169 and 145 bp long) are indicated. *Std*, molecular weight standard.

brain to see whether these are the preferential sites of expression, as we have shown previously for βARK-1 and βARK-2 (5, 11). However, because of the difficulty in obtaining good quality human brain mRNA from surgically excised brain samples, a direct comparison on human tissues turned out to be impossible. To overcome this problem, we studied β-arrestin-1 mRNA expression in bovine tissues including some brain regions (Fig. 4). High levels of β-arrestin-1-specific transcripts were found in bovine PBL as well as in cerebral cortex and cerebellum. In Northern blots of bovine RNA, the band corresponding to the human 3.0-kb band was of higher molecular weight (~4.0 kb, Fig. 4), in keeping with the hybridization pattern reported by others on bovine RNA (6).

The relative abundance of β-arrestin-1A and β-arrestin-1B was studied in various tissues and cell types. In all cases, PCR amplifications generated two products, indicating that both isoforms are expressed (Fig. 5). Similar abundance of the two isoforms was detected in many tissues and cell types, as in

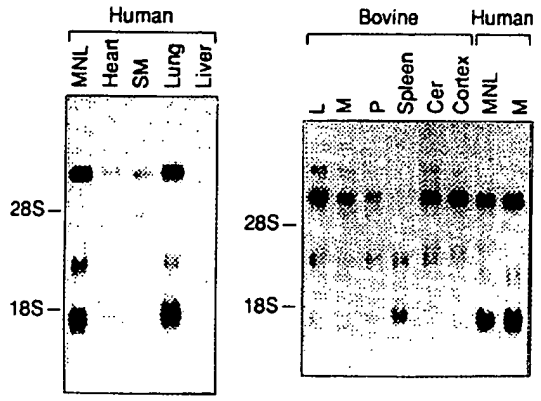

FIG. 4. **Tissue distribution of β-arrestin-1 mRNA.** Total RNA (20 μg) from human MNL, heart, skeletal muscle (*SM*), lung and liver and bovine lymphocytes (*L*), monocytes (*M*), polymorphonuclear leukocytes (*P*), spleen, cerebellum (*Cer*) and cortex was hybridized with a single-stranded DNA probe (bp 684–855) for human β-arrestin-1, labeled as described under "Experimental Procedures." Washed filters were exposed at −80 °C for 48–60 h. In the blot of bovine tissues human MNL and monocytes (*M*) were included for comparison. Data represent two separate experiments.

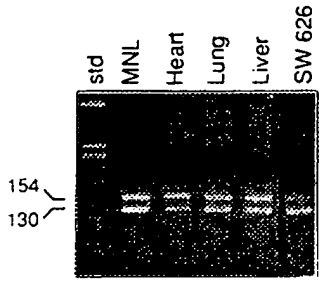

FIG. 5. **PCR analysis of β-arrestin-1A and β-arrestin-1B mRNA from different human tissues and cells.** One μg of total RNA from MNL, heart, lung, liver, and SW626 cells was reverse transcribed with random hexamers. PCR using 0.8 μg each of F4 and R3 was carried out and 18 μl of PCR analyzed by electrophoresis in 4% NuSieve 3:1 agarose gel. The two amplification products of these reactions (154 and 130 bp long) are indicated. *Std*, molecular weight standard.

MNL, liver, lung, skeletal muscle, and adipose tissue (Fig. 5 and data not shown). A prevalence of β-arrestin-1A was found in the heart and in IMR32 cells (a human neuroblastoma cell line, Ref. 5), whereas a prevalence of β-arrestin-1B was seen in SMC and SW626 (a human ovarian carcinoma cell line, Ref. 5) cells (Fig. 5 and data not shown).

A monoclonal antibody directed against a conserved epitope was used to study β-arrestin-1 immunoreactivity. Western blot analysis revealed in all tissues and cells tested one band with an apparent M_r of ~50,000 (Fig. 6), consistent with that reported previously for β-arrestin-1 (7, 8). The intensity of the immunoreactive band was highest in MNL, moderate in the lung and SMC, and barely detectable in the heart (Fig. 6). A second band of immunoreactivity (M_r ~47,000) was revealed in MNL (Fig. 6). As we reported mRNA expression of arrestin in PBL, the molecular weight of this second band suggests that it may be either β-arrestin-2 (8) or arrestin (7). The former possibility is likely to be true, as a second monoclonal antibody, selective for arrestin (A9C6, Ref.19), failed to recognize the 47-kDa band. Direct comparison of β-arrestin-1 immunoreactivity in human MNL with that in rat brain was performed. The signal in MNL was slightly higher than in rat brain (not shown).

Increased β-Arrestin-1 Expression by cAMP—We investi-

gated whether the expression of β-arrestin-1 mRNA is modulated by activation of intracellular signal transduction pathways. For these experiments we did not use PBL, as in preliminary experiments we obtained inconsistent results, probably because of the heterogeneity of cell populations present in PBL. HEC and SMC were used instead. In HEC, β-arrestin-1 mRNA expression was unaffected by a 6-h exposure to the calcium ionophore ionomycin (300 nM) or to the protein kinase C-activating agent phorbol 12-myristate 13-acetate (300 nM) (Fig. 7). By contrast, a 6-h treatment with cholera toxin (1 nM) and forskolin (100 μM) raised β-arrestin-1 mRNA to 419 ± 140% and 300 ± 48% (n = 3) of untreated control, respectively (Fig. 7). Both of these drugs raise intracellular cAMP, by activating the cAMP-generating pathway at different levels: cholera toxin stimulates G_s while forskolin activates adenylylcyclase. Iloprost, a synthetic analog of prostacyclin, which acts through a specific receptor coupled to G_s, also increased β-arrestin-1 mRNA (Fig. 7), although at a lower extent (173% of untreated control, n = 2).

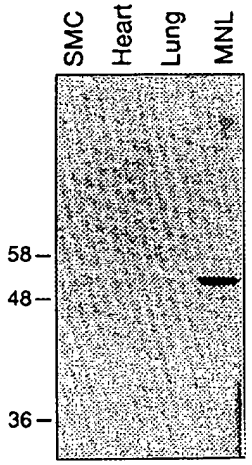

FIG. 6. **β-Arrestin-1 immunoreactivity in human tissues and cells.** 100 μg of cytosolic proteins from SMC, heart, lung, and MNL were prepared, electrophoresed on SDS-polyacrylamide gels, and transferred to nitrocellulose membranes as described under "Experimental Procedures." β-Arrestin-1 immunoreactivity was detected with the F4C1 monoclonal antibody, followed by a peroxidase-coupled second antibody, using 5-chloro-1-naphthol as revealing substrate. Data represent two separate experiments. The positions of molecular mass standards expressed in kilodaltons are indicated.

The effect of cAMP on β-arrestin-1 mRNA was potentiated by okadaic acid, a potent and specific inhibitor of phosphatases 1 and 2A (Fig. 7). Both okadaic acid and forskolin increased β-arrestin-1 mRNA, but the effect was dramatic (~5-fold increase) when the drugs were combined (Fig. 7). Similar results were obtained using iloprost: the combined treatment of HEC with iloprost plus okadaic acid increased the β-arrestin-1 expression by more than 5-fold (not shown). The increase of β-arrestin-1 mRNA levels induced by cAMP was time-dependent, with a peak at around 6–8 h of treatment, whereas mRNA expression returned to the levels of untreated cells after a 20–24 h exposure to forskolin or cholera toxin (Fig. 8).

Parallel experiments in which the specific βARK-1 transcript was probed clearly showed that these treatments do not affect mRNA expression of this kinase (Figs. 7 and 8). Note that in several cases, comparative analysis of β-arrestin-1 and βARK-1 mRNA was done by subsequent hybridization of the same filter with specific probes for the two genes. This means that unchanged βARK-1 mRNA levels provide an internal control for the changes of β-arrestin-1 mRNA.

To see whether the observed effect also occurs in different cells, we measured β-arrestin-1 expression in SMC treated with forskolin (Fig. 9). Our results clearly show that forskolin treatment, which in these cells raised basal intracellular cAMP by approximately 10-fold, increased β-arrestin-1 mRNA levels to 347 ± 45% of untreated control (n = 4). The time course (Fig. 9) was similar to that observed in HEC, with a maximal effect after 8 h of treatment. This effect was also dose-dependent (Fig. 9). The increase of β-arrestin-1 mRNA induced by forskolin was completely prevented by cycloheximide (Fig. 9). Forskolin treatment did not affect βARK-1 mRNA expression in SMC (not shown). An 8-h treatment of SMC with the β-adrenergic agonist isoproterenol (10 μM) increased β-arrestin-1 mRNA levels to 205% of untreated control (not shown).

β-Arrestin-1 immunoreactivity was studied in SMC treated with forskolin (Fig. 10). Western blot analysis of the cytosolic fraction of SMC revealed a single, clearly detectable band of immunoreactivity in all cases (Figs. 6 and 10). This band showed a time-dependent increase in intensity, with a peak after 8-h treatments (264 ± 37% of untreated control, n = 3, Fig. 10).

We studied whether cAMP preferentially increased the expression of either β-arrestin-1 isoform (Fig. 11). The relative abundance of the two isoforms was determined by PCR

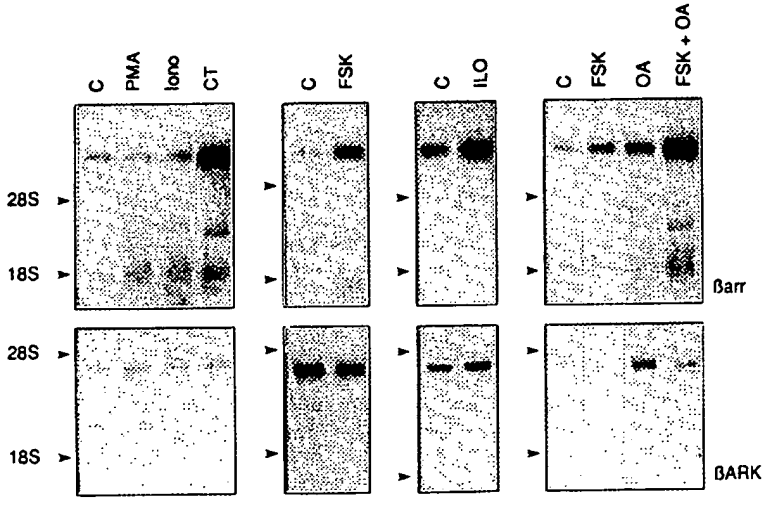

FIG. 7. **β-Arrestin-1 and βARK-1 mRNA expression in HEC exposed to various agents.** Blots of total mRNA (15 μg) from untreated HEC (control, *C*) or HEC treated for 6 h with 300 nM phorbol 12-myristate 13-acetate (*PMA*), 300 nM ionomycin (*Iono*), 1 nM cholera toxin (*CT*), 1 μM iloprost (*ILO*), 100 μM forskolin (*FSK*), 0.5 μM okadaic acid (*OA*), and forskolin plus okadaic acid (*FSK + OA*) were sequentially hybridized with probes specific for β-arrestin-1 (*βarr*) and βARK-1 (*βARK*). Washed filters were exposed at −80 °C for 48–96 h (for β-arrestin-1) and for 18–24 h (for βARK-1). Data represent at least two separate experiments.

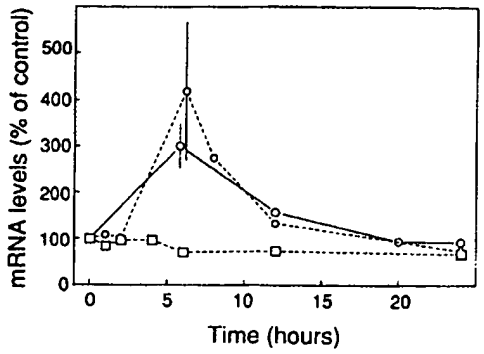

FIG. 8. **β-Arrestin-1 and βARK-1 mRNA expression in HEC treated with cholera toxin and forskolin.** HEC were treated with 1 nM cholera toxin (*filled symbols*) and with 100 μM forskolin (*unfilled circles*) for the indicated times. Total mRNA (15 μg) from untreated and treated HEC was prepared at the indicated times and sequentially hybridized with probes specific for β-arrestin-1 (*circles*) and βARK-1 (*squares*). mRNA expression, quantified by densitometric analysis of autoradiographic films, is expressed as a percentage of the relative untreated control harvested at the same time. Data are means ± S.E. of two to three separate experiments.

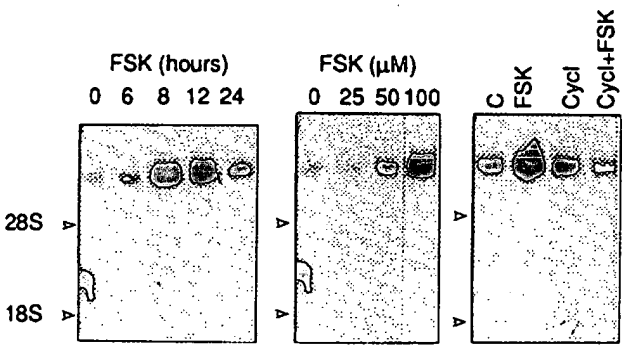

FIG. 9. **Increase of β-arrestin-1 mRNA in SMC treated with forskolin.** *Left panel*, time-dependent increase of β-arrestin-1 mRNA in SMC treated with forskolin. SMC were untreated (*0*) or treated with 100 μM forskolin for the indicated times. *Center panel*, dose-dependent increase of β-arrestin-1 mRNA in SMC treated for 8 h with the indicated doses of forskolin. *Right panel*, cycloheximide prevents the effect of forskolin. SMC were untreated (control, *C*) or treated for 8 h with 100 μM forskolin (*FSK*), 10 μg/ml cycloheximide (*Cycl*), and the two combined (*Cycl+FSK*). In all experiments total mRNA (20 μg) for each condition was hybridized with a single-stranded DNA probe (bp 684–855) for β-arrestin-1. Washed filters were exposed at −80 °C for 36–60 h. Dose and time dependence experiments shown are from the same blot.

on mRNA from SMC exposed to forskolin for 8 h. PCR products separated and visualized by 3:1 NuSieve agarose gels indicated a modified ratio between the two isoforms, β-arrestin-1A being relatively more abundant after the treatment (Fig. 11). To quantify this effect, we performed a Southern blot analysis and measured the relative density of the PCR products on the autoradiographic film by densitometry. At saturation (30 PCR cycles) the ratio of β-arrestin-1B *versus* β-arrestin-1A, which was ~5:1 in control cells, became ~2:1 in treated cells (Fig. 11), showing an increase of the longer isoform. This increase of β-arrestin-1A seems to be preferential rather than selective. In fact Southern blot analysis of the two products during the exponential phase of PCR (20 cycles), which closely reflects the levels of mRNA expression (22), revealed a ~2-fold increase of β-arrestin 1B in forskolin-treated cells compared with control (data not shown). This suggests that both isoforms are increased by cAMP, β-arrestin-1A being more affected.

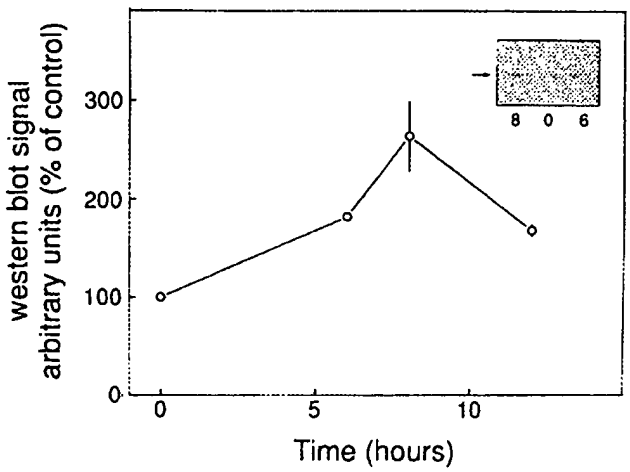

FIG. 10. **Time-dependent increase of β-arrestin-1 immunoreactivity in SMC treated with forskolin.** β-Arrestin-1 immunoreactivity was detected with the F4C1 monoclonal antibody, followed by a peroxidase-coupled second antibody, using 5-chloro-1-naphthol as revealing substrate. β-Arrestin-1 immunoreactivity in treated cells, quantitated by densitometric analysis of Western blots, is expressed as percentage of untreated controls. Data are means ± S.E. of two to three separate experiments. *Inset*, β-arrestin-1 immunoreactivity (*arrowed*) on Western blot after 6 and 8 h of forskolin treatment compared with control (*middle lane*). Different times of treatment are indicated at the *bottom*.

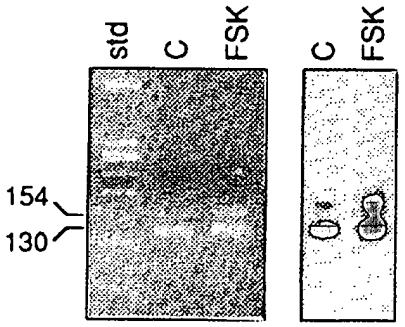

FIG. 11. **PCR analysis of β-arrestin-1A and β-arrestin-1B mRNA in SMC treated with forskolin.** One μg of total RNA from untreated SMC (control, *C*) or SMC treated for 8 h with 100 μM forskolin (*FSK*) was reverse transcribed with random hexamers. PCR using 0.8 μg each of F4 and R3 was carried out and PCR products analyzed by electrophoresis in 4% NuSieve 3:1 agarose gel. *Left panel*, ethidium bromide staining of 18 μl of PCR; the two amplification products of these reactions are indicated. *Std*, molecular weight standards. *Right panel*, 1 μl of PCR was blotted and probed as described. The two amplification products of these reactions are revealed on autoradiographic film after Southern blot analysis. Shown are the results of two independent experiments.

DISCUSSION

The cDNA of human β-arrestin-1 was cloned and sequenced. The sequence is very similar to that of bovine β-arrestin-1 (6): the overall amino acid identity is 98.6%. This appears as an unpredictably high level of interspecies conservation which indicates that any domain of β-arrestin-1 may have a relevant functional role. A second form of β-arrestin (called β-arrestin-2) was recently cloned in human (9) and rat (8). The amino acid homology (Fig. 1) of human β-arrestin-1 with human β-arrestin-2 (9) and arrestin (12) is 84 and 68%, respectively.

We report here for the first time the existence of two isoforms of β-arrestin-1. We named these two molecular forms, defined by the presence or absence of an 8-amino acid

sequence within the protein, β-arrestin-1A and β-arrestin-1B, respectively. The two isoforms are likely to be generated by alternative mRNA splicing, as Southern blot analysis of human genomic DNA indicated the existence of a single gene for β-arrestin-1. Both β-arrestin-1A and β-arrestin-1B mRNA are expressed in all tissues and cells examined, and in some cases their relative levels differ significantly. Two isoforms of β-arrestin-1 were found in RNA from bovine PBL, indicating that this alternative mRNA splicing is conserved in human and bovine.

The sequence of β-arrestin-1 encompassing the site of alternative splicing was aligned with the corresponding sequence of β-arrestin-2 and arrestin, the two other known members of the arrestin/β-arrestin gene family (Fig. 1). It appeared that the 24-bp sequence of human and bovine β-arrestin-1, which is the site of alternative mRNA splicing (bp 999–1022), corresponded exactly to one gap in the sequence of β-arrestin-2 and to an exon (exon 13) in the genes of both human (23) and mouse (24) arrestin. These observations suggested to us that this region may represent a site of alternative splicing in other members of the arrestin/β-arrestin gene family. This possibility was tested using a PCR-based approach.

In all tissues and cells considered, PCR analysis of human β-arrestin-2 always revealed a single specific product, consistent with the presence of one form of β-arrestin-2 mRNA, in which the 24-bp sequence of interest is lacking. By contrast, the analysis of arrestin mRNA revealed several unexpected findings: (i) only one isoform of arrestin was found in the retina; (ii) the presence of arrestin mRNA was documented in some, but not in all, of the non-retinal tissues examined; (iii) two isoforms of arrestin, derived from alternative mRNA splicing, are expressed in PBL. These results confirm and extend previous evidence of arrestin being expressed in non-retinal tissues (25). We found mRNA expression of arrestin in bovine cerebellum and cortex but not in liver and heart. Furthermore, we provide the first evidence of the existence of two alternatively spliced isoforms of arrestin in bovine PBL. By analogy with β-arrestin-1 we named these two isoforms arrestin A and arrestin B in the presence or absence of the 24-bp/8-amino acid sequence, respectively.

Arrestin (also called S-antigen) is the major immunogenic determinant of autoimmune uveitis (26). The expression of arrestin in PBL may shed new light on the pathogenesis of autoimmune uveitis secondary to general diseases, such as rheumatoid arthritis. We can hypothesize that in the course of inflammation the lysis of reacting PBL may determine a release of arrestin, thus starting the autoimmune reaction. Interestingly, the 8 alternatively spliced amino acids of arrestin represent the core of a potent, newly identified immunopathogenic epitope that is flanked by potent immunoproliferative determinants (27). Only the immunogenic stimulus of this region seems to be exposed by arrestin A. We speculate that arrestin B may have the immunoproliferative determinants exposed. If so, the concomitant release of the two isoforms from PBL may provide both immunopathogenic and immunoproliferative activation of T-cells, ultimately leading to uveitis (28). Additionally, it has been shown that arrestin and β-arrestin are recognized by autoantibodies obtained from patients with multiple sclerosis.[2] This common chronic neurologic disease is characterized by demyelination throughout the central nervous system. Although the presence of these antibodies may be a secondary effect, it is possible that they are also involved in the pathogenesis of the disease.

[2] H. Oghuro, S. Chiba, Y. Igarashi, H. Matsumoto, T. Akino, and K. Palczewski, manuscript in preparation.

The presence of the same alternative mRNA splicing in two out of three members of the arrestin/β-arrestin gene family suggests that this is generated by a similar, highly conserved mechanism. A "cassette exon" mechanism (29) of alternative splicing can be postulated, as the spliced sequence is encoded by a separate exon (exon 13) in the arrestin genes (23, 24). In the arrestin/β-arrestin gene family, gene duplication events resulting in different arrestin-like proteins seemed to be the only mechanism generating protein diversity (6, 8, 12). The three members of this family identified so far are in fact individual products of different genes. In the present study, we provide evidence for the use of an alternative mRNA splicing mechanism to generate further diversity within the arrestin/β-arrestin gene family. Diversity in function is conceivable, as the alternative splicing site is located near the C-terminal region of these proteins, which represents a functionally relevant domain (6, 30, 31). Sequence differences at the C termini between members of the arrestin/β-arrestin gene family may reflect the specificity for tight binding of these regulatory proteins to selected G-protein-coupled receptors. A functional role for the alternatively spliced isoforms is also suggested by tissue selectivity of their expression. This is particularly true for arrestin, whose shorter form seems to be expressed only in PBL. The preferential regulation of human β-arrestin-1A by cAMP (see below) further supports this view. However, the possible functional differences between molecular isoforms, if any, remain to be demonstrated.

The expression of β-arrestin-1 was investigated in different tissues and cells. High levels of β-arrestin-1 mRNA and immunoreactivity were found in human PBL. This finding is of particular interest, as it extends our previous evidence suggesting a major role of βARK-induced desensitization in regulating receptor-mediated immune functions (5, 11). According to this hypothesis, an abundant presence of β-arrestin, which is an essential cofactor for βARK-induced desensitization to be fully effective, was expected in these cells. This point is indeed demonstrated by the present findings.

Our investigation documents for the first time that β-arrestin-1 expression is regulated by intracellular cAMP. The levels of β-arrestin-1 mRNA were increased in both HEC and SMC treated with different agents that raise intracellular cAMP. The peak of the effect occurred after 6–8 h of treatment. The increased mRNA levels induced by cAMP resulted in an increased expression of the protein, as β-arrestin-1 immunoreactivity was 2–3-fold higher in SMC exposed to forskolin for 8 h, compared with untreated controls.

Several genes are regulated by intracellular cAMP. They can be divided into two general categories that probably reflect different mechanisms of regulation (32). The first group of are those that are rapidly regulated by cAMP, within minutes, and the regulation is usually insensitive to cycloheximide. These genes have relatively short lived mRNAs (minutes *versus* hours). By contrast, in the second group of genes the mRNA is increased only after several hours of cAMP treatment. To date, the genes that are rapidly regulated by cAMP are the most extensively characterized, whereas only few examples of the second group have been reported (32). Our present work provides three types of evidence indicating that the β-arrestin-1 gene belongs to the second group: (i) the half-life of β-arrestin-1 mRNA is relatively long (around 7 h, Ref. 18); (ii) the increase of β-arrestin-1 mRNA was only observed after several hours of treatment; (iii) cycloheximide completely prevented this effect. The last point strongly indicates that a newly synthesized protein(s) plays a major role in the cAMP-induced increase of β-arrestin-1 mRNA. The need of newly synthesized protein(s) may also explain why several

hours are required to induce this effect.

The mechanism by which cAMP increases β-arrestin-1 expression likely involves the activation of cAMP-dependent protein kinase (protein kinase A). The experiments with okadaic acid, a potent and specific inhibitor of phosphatases 1 and 2A (33), further support this hypothesis. In HEC, okadaic acid alone induced a moderate increase of the β-arrestin-1 mRNA, but it potentiated dramatically the effect of forskolin and iloprost. The mechanism suggested by these data is a protein kinase A-mediated induction of gene expression, which is attenuated by intracellular phosphatases. Such a mechanism has been demonstrated directly for other genes, although in a different experimental model (34). Further work will be needed to clarify whether transcriptional activation or an increase of mRNA stability or both are responsible for the increased mRNA levels of β-arrestin-1.

Several mechanisms have been identified by which cells can attenuate persistent stimulation of cAMP-generating pathways (1–3), and many of them are mediated by activation of protein kinase A (3). Since β-arrestin-1 is able to quench the receptor-mediated intracellular signaling, the increase in its expression is likely to represent a novel regulatory mechanism that goes along this way. The fact that β-arrestin-1, but not βARK-1, is regulated by cAMP indicates that different proteins that work as cofactors in desensitizing receptors are regulated in different manners. In particular, to enhance the activity of β-arrestin-1, for which a ~1:1 stoichiometric mechanism of receptor desensitization has been demonstrated (7), an increase in protein concentration may be needed. Such an increase is documented by our work. For βARK-1, which enzymatically regulates the receptor, the activity may be enhanced by mechanisms other than modulation of RNA levels.

Receptor desensitization by βARK/β-arrestin is a multistep phenomenon. βARK phosphorylates the receptor only when it is occupied by its own agonist, and then β-arrestin leads to complete desensitization. This represents the classical paradigm of homologous desensitization (35). On the other hand, our data show that agonists acting through different cAMP-generating receptors (as iloprost and isoproterenol) increase intracellular β-arrestin-1, which in turn may regulate different G-coupled receptors. This represents the classical paradigm of heterologous desensitization (35). Our findings thus outline a novel mechanism whereby one receptor may regulate other receptors' responses (heterologous desensitization) by potentiating their own signal termination mechanisms (homologous desensitization).

In conclusion, the present paper provides several new clues on β-arrestin-1. The finding of alternatively spliced isoforms of β-arrestin-1 and arrestin documents a novel mechanism to generate diversity within the arrestin/β-arrestin gene family. The abundant expression of β-arrestin-1 in PBL further supports our previous suggestion of a major role of the βARK/β-arrestin system in regulating receptor-mediated immune functions. Furthermore, the increase of β-arrestin-1 (but not βARK-1) expression induced by intracellular cAMP suggests a new mechanism by which the regulation of receptor-mediated responses can be regulated.

Acknowledgments—We thank Dr. T. Vago, Prof. A. M. Caracino, and Prof. P. Musiani for providing surgical specimens; Drs. R. Buccione and F. Facchiano for assistance in Western blot experiments; Dr. M. S. Lombardi for assistance in sequence analysis; G. D'Alessandro for oligonucleotide synthesis; and R. Bertazzi and S. Menna for expert assistance in the preparation of figures. We are grateful to Dr. L. Di Filippo of Centro Trasfusionale at Lanciano Hospital for providing buffy coats.

REFERENCES

1. Hausdorff, W. P., Caron, M. G., and Lefkowitz, R. J. (1990) *FASEB J.* **4**, 2881–2889
2. Palczewski, K., and Benovic, J. L. (1991) *Trends Biochem. Sci.* **16**, 387–391
3. Collins, S., Caron, M. G., and Lefkowitz, R. J. (1992) *Trends Biochem. Sci.* **17**, 37–39
4. Benovic, J. L., De Blasi, A., Stone, W. C., Caron, M. G., and Lefkowitz, R. J. (1989) *Science* **246**, 235–240
5. Chuang, T. T., Sallese, M., Ambrosini, G., Parruti, G., and De Blasi, A. (1992) *J. Biol. Chem.* **267**, 6886–6892
6. Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1990) *Science* **248**, 1547–1550
7. Lohse, M. J., Andexinger, S., Pitcher, J., Trukawinski, S., Codina, J., Faure, J. P., Caron, M. G., and Lefkowitz, R. J. (1992) *J. Biol. Chem.* **267**, 8558–8564
8. Attramadal, H., Arriza, J. L., Aoki, C., Dawson, T. M., Codina, J., Kwatra, M. M., Snyder, S. H., Caron, M. G., and Lefkowitz, R. J. (1992) *J. Biol. Chem.* **267**, 17882–17890
9. Rapoport, B., Kaufman, K. D., and Chazenbalk, G. D. (1992) *Mol. Cell. Endocrinol.* **84**, R39–R43
10. Lorenz, W., Inglese, J., Palczewski, K., Onorato, J. J., Caron, M. G., and Lefkowitz, R. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 8715–8719
11. Parruti, G., Ambrosini, G., Sallese, M., and De Blasi, A. (1993) *Biochem. Biophys. Res. Commun.* **190**, 475–481
12. Yamaki, K., Tsuda, M., and Shinohara, T. (1988) *FEBS Lett.* **234**, 39–43
13. Shinohara, T., Dietzschold, B., Craft, C. M., Wistow, G., Early, J. J., Donoso, L. A., Horwitz, J., and Tao, R. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 6975–6979
14. De Blasi, A., Cotecchia, S., Fratelli, M., and Lipartiti, M. (1986) *J. Lab. Clin. Med.* **107**, 86–94
15. Gilliland, G., Perrin, S., Blanchard, K., and Bunn, H. F. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 2725–2729
16. Yagle, M. K., Parruti, G., Xu, W., Ponder B. A. J., and Solomon, E. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 7255–7259
17. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1990) *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, New York
18. Parruti, G., Ambrosini, G., Sallese, M., and De Blasi, A. (1993) *J. Rec. Res.* **13**, 609–618
19. Knospe, V., Donoso, L. A., Banga, J. P., Yue, S., Kasp, E., and Gregerson, D. S. (1988) *Curr. Eye Res.* **7**, 1137–1147
20. Balconi, G., and Dejana, E. (1986) *Med. Biol.* **64**, 231–245
21. Peracchia, F., Polenfarutti, N., Colotta, F., and Mussoni, L. (1989) *Biochem. Biophys. Res. Commun.* **160**, 977–981
22. Siebert, P. D., and Larrick J. W. (1992) *Nature* **359**, 557–558
23. Yamaki, K., Tsuda, M., Kikuchi, T., Chen, K. H., Huang, K. P., and Shinohara, T. (1990) *J. Biol. Chem.* **265**, 20757–20762
24. Tsuda, M., Kikuchi, T., Yamaki, K., and Shinohara, T. (1991) *Eur. J. Biochem.* **200**, 95–101
25. Breitman, M. L., Tsuda, M., Usukura, J., Kikuchi, T., Zucconi, A., Khoo, W., and Shinohara, T. (1991) *J. Biol. Chem.* **266**, 15505–15510
26. Donoso, L. A., Merryman, C. F., Shinohara, T., Dietzschold, B., Wistow, G., Craft, C., Morley, W., and Henry, R. T. (1986) *Curr. Eye Res.* **5**, 995–1004
27. Merryman, C. F., Donoso, L. A., Zhang, X., Heber-Katz, E., and Gregerson, D. S. (1991) *J. Immunol.* **146**, 75–80
28. Fling, S. P., Donoso, L. A., and Gregerson, D. S. (1991) *J. Immunol.* **147**, 483–489
29. Breitbart, R. E., Andreadis, A., and Nadal-Ginard, B. (1987) *Annu. Rev. Biochem.* **56**, 467–495
30. Schleicher, A., Kuhn, H., and Hofmann, K. P. (1989) *Biochemistry* **28**, 1770–1775
31. Palczewski, K., Pulvermuller, A., Buczylco, J., and Hofmann, K. P. (1991) *J. Biol. Chem.* **266**, 18649–18654
32. Roesler, W. J., Vandenbark, G. R., and Hanson, R. W. (1988) *J. Biol. Chem.* **263**, 9063–9066
33. Sassa, T., Richter, W. W., Uda, N., Suganuma, M., Suguri, H., Yoshizawa, S., Hirota, M., and Fujiki, H. (1989) *Biochem. Biophys. Res. Commun.* **159**, 939–944
34. Hagiwara, M., Alberts, A., Brindle, P., Meinkoth, J., Feramisco, J., Deng, T., Karin, M., Shenolikar, S., and Montminy, M. (1992) *Cell* **70**, 105–113
35. Sibley, D. R., and Lefkowitz, R. J. (1985) *Nature* **317**, 124–129

May 5, 1993 VOLUME 268 NUMBER 13

ISSN 0021-9258
JBCHA3 268(13) 9157-9936 (1993)

THE Journal of Biological Chemistry

Published by the American Society for Biochemistry and Molecular Biology

FOUNDED BY CHRISTIAN A HERTER

AND SUSTAINED IN PART BY THE CHRISTIAN A HERTER MEMORIAL FUND